**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

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| Application Number | : 10/713,008 | Confirmation No. | 5744 |
| Applicants | : Masaki IKEDA et al. | | |
| Filed | : November 17, 2003 | | |
| Title | : METHODS FOR PROLIFERATING TERMINAL DIFFERENTIATED CELLS AND RECOMBINANT VECTORS THEREFOR | | |
| TC/Art Unit | : 1636 | | |
| Examiner: | : Tara L. GARVEY | | |
| Docket No. | : 64517.000002 | | |
| Customer No. | : 21967 | | |

Commissioner for Patents
P.O. Box 1450
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**DECLARATION UNDER 37 C.F.R. § 1.132**

Sir,

I, Uichi Koshimizu, Ph.D., declare that:

1) I am the manager and chief researcher at Asubio Pharma Co., Ltd. I have been employed by Asubio Pharma Co., Ltd. for 6 years as a researcher and have been involved in the study of regenerative medicine and gene therapy.

2) I have received a Bachelor of Sciences degree in Agriculture from the Meiji University, Kanagawa, Japan, in 1988 and a Doctor of Philosophy degree in Medicine from the Osaka University Medical School, Osaka, Japan, in 1994.

3) I have been associated with research in the field of cell biology, molecular biology, biochemistry, developmental and regenerative biology, gene therapy research and cardiovascular research for approximately 15 years, and am an author of more than 70 publications including peer-reviewed papers, review articles, book chapters, and patent specifications. A recitation of some of these publications, together with details of my education, are given in my *curriculum vitae* which is attached as Exhibit A.

4) Based on the academic training and professional experience, I consider myself a person of ordinary skill in the technology of regenerative biology, gene therapy research and cardiovascular research, and I was such a person in 2001 when the U.S. Patent Application Serial Number 10/713,008 (hereinafter referred to as "the '008 application") was filed.

5) At the request of Asubio Pharma Co., Ltd., the exclusive licensee of the above-identified application, I have reviewed the following documents:

a) the '008 application;

b) the Final Office Action mailed on October 6, 2006 and the references cited therein, in the '008 application;

c) the Amendment and Response Under 37 C.F.R. § 116 filed on March 6, 2007 and the references cited therein, in the '008 application

d) the Advisory Action mailed on March 30, 2007, in the '008 application.

6) It is my understanding that claims 1, 2, 4, 6, and 16-19 of the '008 application were rejected because the specification allegedly does not reasonably provide enablement for the scope of the claimed subject matter. I have been asked to comment on whether the specification of the '008 application is enabling for a method of proliferating cardiomyocytes comprising introducing an adenoviral vector comprising a D-type cyclin and cyclin dependent kinase gene into cardiomyocytes *in vivo*.

7) I have conducted experiments demonstrating that the introduction of adenoviral vectors expressing a D-type cyclin, CDK4 and a nuclear localization signal into adult rat cardiomyocytes *in vivo* proliferates the cardiomyocytes. These experimental results are set forth in the following paragraphs.

Preparation of Recombinant Adenovirus

8) Adenovirus vectors, each carrying the CDK4 gene or the cyclin D1 gene with the nucleotide sequence encoding the nuclear localization signal (NLS) tagged thereto

(D1NLS) were prepared, using a recombinant adenovirus preparation kit (Adenovirus Expression Vector Kit; TakaRa Bio).

9) The plasmid carrying the CDK4 gene, pCMV-CDK4, (supplied by Dr. Sander van den Heuvel [Massachusetts General Hospital Cancer Center; USA]; van den Heuvel, *et al.*, Science 262:2050, 1993) was digested with BamHI, to prepare a murine CDK4 cDNA fragment. Both ends of the cDNA fragment were blunted using T4 DNA polymerase. Following a protocol attached to the Adenovirus Expression Vector Kit, the blunted fragment was inserted into the *Swa*I site of a cosmid pAxCAwt, to prepare a cosmid pAd-CDK4. Continuously, the transfection of the cosmid together with the restriction enzyme-treated DNA-TPC (terminal peptide complex) derived from the genome DNA of human adenovirus type 5 into the 293 cells derived from human embryonic kidney cells allowed the preparation of a recombinant adenovirus, hereinafter referred to as "Ad-CDK4."

10) A plasmid carrying the D1NLS gene was constructed by conjugating the murine cyclin D1 cDNA fragment derived from pRSV-cyclin D1 (Matsushime, *et al.*, Cell 65:701, 1991) with pEF/myc/nuc (Invitrogen)-derived NLS. The plasmid pEF/myc/nuc was digested with restriction enzymes *Nco*I and *Xho*I, to prepare a first DNA fragment containing the NLS sequence. Then, the plasmid pRSV-cyclin D1 was digested with a restriction enzyme *Nco*I, to prepare a second DNA fragment containing the cyclin D1 sequence. Then, PCR was performed using the plasmid pRSV-cyclin D1 as template and also using the following two types of primers, to prepare a third DNA fragment encoding the C-terminal cyclin D1 cDNA.

5′-primer: 5'-ACCCTCCATGGTAGCTGCTGGGA3'

3′-primer: 5'-TGATCTCGAGGTCGATGTCCACATCTCGCACGT-3'

Using T4 DNA ligase, these three types of DNA fragments were ligated together, to construct a plasmid carrying a nucleotide sequence encoding SV40 T antigen-derived NLS triplicately on the side of the C terminus of the murine cyclin D1 cDNA. A DNA fragment excised from the plasmid with restriction enzymes *Pma*CI and *Sma*I was inserted in the *Swa*I site of the cosmid pAxCAwt. The resulting cosmid (pAd-D1NLS)

and the restriction enzyme-treated DNA TPC were transfected into the 293 cell, to prepare a recombinant adenovirus, hereinafter referred to as "Ad-D1NLS."

11) Ad-CDK4 and Ad-D1NLS were constructed in such a manner that the individual genes inserted may be expressed under the regulation of CAG promoters (CMV enhancer, chick β-actin promoter, and the polyA sequence of rabbit β-globin gene). Therefore, the inserted genes can be highly expressed in mammalian cells.

12) Continuously, high titers of virus solutions of the individual recombinant viruses were prepared. 4 μg each of the cosmids (pAd-CDK4 and pAd-D1NLS) were mixed with 2.5 μl of the restriction enzyme-treated DNA-TPC attached to the recombinant adenovirus preparation kit. The individual mixtures were separately transfected into the 293 cell cultured in a culture dish (diameter of 60 mm) by lipofection method using FuGENE™ 6 Transfection Reagent (Roche). On the following day, the cells were detached, and recovered cell suspensions were separately inoculated again in a culture plate (96 well) coated with collagen. After 7 to 15 days, the virus grew and the cells were killed in several wells. From each wells in which cells completely died, the culture medium was aseptically collected into a sterilized tube, freezing and thawing was repeated 6 times, and centrifuged at 500rpm for 5 minutes. The supernatants were stored as a primary virus stock solution at -80°C. 10 μl of the primary virus stock solution was infected into the 293 cells cultured in a culture plate (24 well) coated with collagen. The culture medium in a well containing killed cells in 3 to 4 days was aseptically transferred into a sterile tube, and freezing and thawing was repeated 6 times and was centrifugated at 5000 rpm for 5 minutes to recover the resulting supernatant and was stored as a secondary virus stock solution at -80°C. 15 μl of the secondary virus stock solution was infected into the 293 cells cultured in a culture flask (25 $cm^2$) coated with collagen. The culture medium after 3 to 4 days was aseptically transferred into a sterile tube, and the virus was released from cells by freezing and thawing or homogenizing cells with a sealed sonicator. The supernatant resulting from centrifugation (3000 rpm, 10 minutes, 4°C) was stored as a third virus stock solution at -80°C. 50 μl of the third virus stock solution was infected into the 293 cells cultured in a culture flask (75 $cm^2$) coated with collagen. The culture medium was aseptically transferred into a sterile tube, and the virus

was released from cells by freezing and thawing or homogenizing cells with a sealed sonicator. The supernatant resulting from centrifugation (3000 rpm, 10 minutes, 4°C) was stored as a fourth virus stock solution at -80°C. The titer of the fourth virus solution was determined by plaque assay using 293 cells. The titer was constantly within a range of $10^9$ to $10^{11}$ pfu/mL, with no exception. The fresh virus number to be infected per cell is expressed as multiplicity of infection, hereinafter referred to as "moi." In other words, one virus particle infected per one cell is expressed as moi = 1.

D1NLS + CDK4 Gene Expression Promotes Cell Cycle and Proliferation of Adult Rat Cardiomyocytes *In Vivo*.

13) To confirm the effect of the expression of D1NLS + CDK4 genes on the proliferation of cardiomyocytes *in vivo*, and that such gene expression *in vivo* has a therapeutic effect on damaged cardiomyocytes, an experiment was performed using a rat myocardial ischemia and reperfusion model.

14) The model was prepared according to the method by Dairaku, *et al.* (Circ. J. 66:411, 2002). A male Wistar rat (8 weeks of age) was anesthetized *via* intraperitoneal administration of sodium pentobarbital (Nembutal: Dainippon Pharmaceutical Co., Ltd.) (55 mg/kg). Subsequently, the rat received thoracotomy under artificial respiration, to expose the heart. Continuously, left coronary artery was ligated with a suturing thread with a needle No. 5-0. Then, the rat was left to stand for 30 minutes. The ligature was released, to allow blood flow to be reperfused (reperfusion). As a control, an animal treated only with threading the coronary artery was used as a sham operation group (referred to as Sham group hereinafter).

15) The adenovirus vector (Ad-D1NLS, Ad-CDK4, and Ad-LacZ) was introduced into the heart during a period of 25 to 30 minutes post-ischemia. A high-titered adenovirus solution ($1 \times 10^9$ pfu/mL) was directly injected into the cardiac muscle layer in the ischemic center and in the periphery thereof at 50 μl each per one site in total of 5 sites (the total volume of 250 μl), using a 30G injection needle.

16) An animal group injected with a mixture solution of Ad-D1NLS ($1 \times 10^9$ pfu) and Ad-CDK4 ($1 \times 10^9$ pfu) is hereinafter referred to as "D1NLS group." As a negative control group, an animal group injected with Ad-LacZ ($2 \times 10^9$ pfu) was arranged and is hereinafter referred to as "Cont group." No injection of any adenovirus solution was done in the Sham group. After closing the chest, the rat was awakened from anesthesia, and then fed under general feeding conditions.

17) As an indicator of the necrosis of cardiac muscle following ischemia and disordered reperfusion, the cardiac muscle troponin T (cTnT) value in plasma was assayed (O'brien, *et al.*, Lab. Anim. Sci. 47:486, 1997; Morimoto, *et al.*, J. Pharmacol. Sci. 91:151 [Suppl. 1], 2003). Two hours after reperfusion, blood was drawn out from the fundus and centrifuged to recover plasma. The cTnT value was assayed, using a cardiac reader (Roche Diagnostics). Consequently, it was observed that the cTnT value after ischemia and reperfusion was greatly increased in the Cont group and the D1NLS group, compared with the Sham group (Sham group: 0.2 ± 0.0 ng/mL, Cont group: 8.8 ± 0.5 ng/mL, D1NLS group: 9.1 ± 0.7 ng/mL). There was no difference in the cTnT value between the D1NLS and Cont groups, suggesting that a similar level of the necrosis of cardiac muscle was induced.

18) At 4 and 7 days after reperfusion/adenovirus infection, hearts were resected, fixed in a 10 % neutrally buffered formalin solution, embedded in paraffin, and sectioned at an interval of 2 mm in each sample along the cross-sectional direction. Subsequently, sections were reacted with several antibodies against markers specific for cardiomyocytes (Troponin I, Tropomyosin), cell growth (Ki67), mitosis (phosphorylated histone H3; H3P), or cytokinesis (Aurora B, Survivin). Immune complexes were detected with Alexa 488, Alexa 568 (Molecular Probes), or Cy5 (Jackson), and observed under a fluorescence microscope.

19) In the Cont (LacZ) group, the percentage of LacZ positive cardiomyocytes in infarct and border zones at 4 days after reperfusion/adenovirus infection was 5%, indicating the efficiency of adenovirus infection in the injected area, and the expression lasts until 7 days after manipulation. Under this condition, it was then determined

whether Ki67-positive cardiomyocytes were observed or not. Ki67 is a marker for proliferating cells, highly expressed from G1, S, G2, prophase, to metaphase, and in hardly detectable in anaphase and telophase. In the Cont group, Ki67-positive cardiomyocytes were hardly detectable at 4 days after the manipulation ("LacZ" in Fig. 1). In contrast, a certain number of Ki67-positive cardiomyocytes were apparently observed in D1NLS group at day 4, and these cells were also positive for a myc-tag, fused to the C-terminal domain of cyclin D1NLS ("D1NLS" in Fig.1), indicating that cardiomyocytes expressing the D1NLS transgene expressed Ki67. Proportions of Ki67 positive cells in myc-tag positive cells were 48.0 ± 3.8 %. Proportions of Ki67-positive cardiomyocytes among total cells in infarct and border zones were 1.95 ± 0.35 % in D1NLS group, but less than 0.1% in Cont group.

20) Next, to determine whether adult cardiomyocytes can undergo cell division *in vivo*, the presence of H3P-positive cardiomyocytes at 4 days after manipulation was investigated. H3P-positive cardiomyocytes with characteristics from early prophase through telophase were identified in D1NLS group, whereas no mitotic cells were found in Cont group, suggesting D1NLS+CDK4 expression promoted karyokinesis of adult cardiomyocytes *in situ* (Fig. 2). Quantitative analysis showed that the percentages of H3P-positive cardiomyocytes were 0 ± 0.0 % and 0.24 ± 0.07 % for Cont and D1NLS groups, respectively. H3P-positive cardiomyocytes were also observed in D1NLS group at 7 days after operation. Furthermore, in D1NLS group, cardiomyocytes at anaphase were also identified, expressing Aurora B in a cleavage furrow, and in some cardiomyocytes, Aurora B and Survivin were detected on the midzone between two daughter cells generated (Fig. 3), indicating that these cells were undergoing cytokinesis. It is important to note that these daughter cells were also positive for tropomyosin, showing that they retained the phenotype of cardiomyocytes. From this data, it is concluded that D1NLS+CDK4 gene expression *in vivo* cause cell division of adult cardiomyocytes *in situ* to generate new functional daughter cells.

<u>D1NLS + CDK4 Gene Expression Reduce Infarct Size and Prevent Cardiac Dysfunction and Heart Failure</u>

21) Six weeks after reperfusion, cardiac functions were measured by tomography (B mode method) and the M mode method displaying tomography over time, using an ultrasonography apparatus (power Vision 8000: Toshiba Medical). Ketamine (Ketaral: Sankyo) and xylazine (Sigma) were intraperitoneally administered to a rat for anesthesia. Using a linear probe of 15 MHz, the left ventricular short-axis papillary muscle was measured by the M mode method, to measure the left ventricular end-diastolic dimension (LVDD) and the left ventricular end-systolic dimension (LVDS) to calculate the fractional shortening of left ventricular inner diameter (FS).

Fractional Shortening of left ventricular inner diameter (FS)

= (end-diastolic dimension – end-systolic dimension)/ end-diastolic dimension × 100 (%)

22) Echocardiographical analysis revealed that LVDD, LVDF, and FS of the Cont/LacZ group was smaller than that of the Sham group. By contrast, FS of D1NLS group was significantly higher than that of the Cont group, suggesting that the expression of D1NLS+CDK4 expression protected ischemic hearts from left ventricular dysfunctioning (Table 1). In addition, in D1NLS group, the "fractional area change of left ventricular," which was used as an indicator of the systolic function, was at larger values than in the Cont group. In D1NLS group, the "E/A value," which was calculated from left ventricular blood flow influx at early diastolic stage (E) and atrium systolic stage (A) by Doppler method, was not statistically significant, but apparently smaller than in the Cont group (Sham group: 2.30 ± 0.25, Cont group: 5.49 ± 0.86, D1NLS group: 3.69 ± 0.68: n = 9 to 12).

23) Similar analysis of hemodynamics showed that heart failure developed 6 weeks after coronary ligation in the Cont/LacZ group, as indicated by attenuation of the maximum and minimum rates of change in left ventricular pressure (dP/dt), increase in the left ventricle end-diastolic pressure (LVEDP) and the left ventricle end-diastolic volume index (LVEDV), and the right-shifted passive pressure-volume curve. These parameters were greatly improved in the D1NLS group as compared to the Cont group.

24) Next, the level of cardiac infarction in the heart 6 weeks post-ischemia and reperfusion was examined. The resected heart was fixed in a 10 % neutrally buffered formalin solution, embedded in paraffin, prepared into 6 sections at an interval of 2 mm in each sample along the cross-sectional direction, and stained with Masson Trichrome to visualize the area with cardiac infarction. Then, the infarct area was measured using an image analysis software (Lumina vision: Mitsuya Shoji). With reference of the report of Jain, *et al.* (Circulation 103:1920, 2001), the infarct area was determined by measuring the whole peripheral length of the inner membrane of left ventricle, the whole peripheral length of the outer membrane thereof, the peripheral length of the cicatrisation on the side of the inner membrane of left ventricle and the peripheral length of the cicatrisation on the side of the outer membrane thereof to calculate the infarct area according to the following formula:

Infarct area = [(the peripheral length of the cicatrisation on the side of the inner membrane of left ventricle + the peripheral length of the cicatrisation on the side of the outer membrane thereof)/ (the whole peripheral length of the inner membrane + the whole peripheral length of the outer membrane)] × 100 (%).

25) Compared with the Cont group, the infarct area in the D1NLS group was significantly reduced (Fig. 4).

26) Based on the above results, it was confirmed that the introduction of an adenoviral vector comprising a D-type cyclin and cyclin dependent kinase gene into cardiomyocytes *in vivo* proliferates the cardiomyocytes. Furthermore, based on the above results, it was confirmed that expression of D1NLS and CDK4 genes had protective effects on cardiac dysfunction and heart failure.

27) In my opinion, a person of skill in the art would understand that the '008 application teaches one of skill in the art to carry out a method for proliferating cardiomyocytes comprising introducing an adenoviral vector comprising a D-type cyclin and cyclin dependent kinase gene into cardiomyocytes *in vivo*.

28) I declare further that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true; and further

that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

I declare under penalty of perjury that the foregoing is true and correct.

Executed on June 12, 2007.

Declarant's Signature: Yuichi Ideshimizu

Home Address:

Hyakuyama 15-1, Shimamoto-cho, Mishima-gun, Osaka, Japan

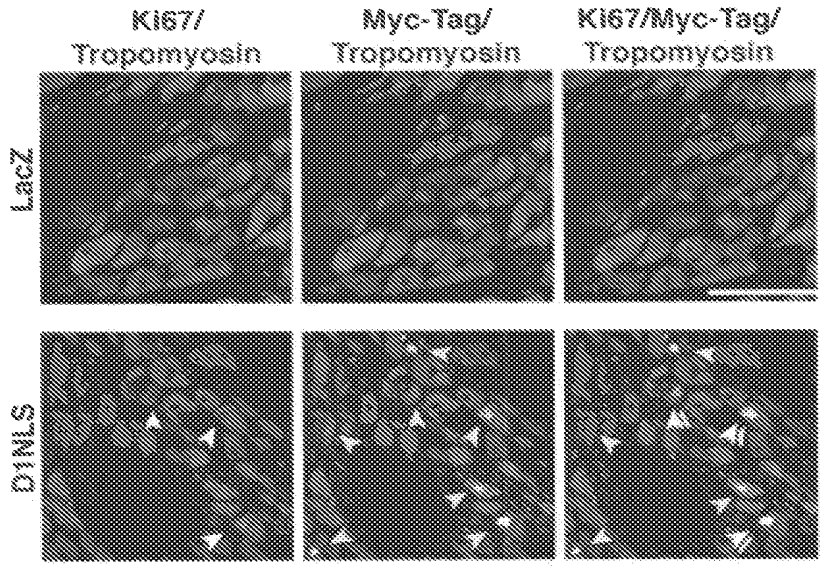


**Fig. 1 Immunostaining of Ki67-postitive cardiomyocytes *in vivo*.**

The expression of Ki67 or myc-tag-D1NLS in cardiac cells injected with adenoviruses for Cont (LacZ) or D1NLS+CDK4 (D1NLS) was examined at day 4 after manipulation. Ki67 (green); Tropomyosin (red); myc-tag (white); DAPI (blue). A scale bar represents 100 $\mu$m.

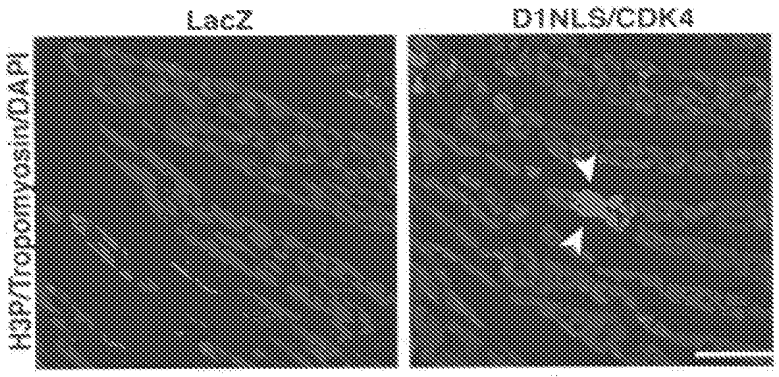


**Fig. 2 Immunostaining of H3P-postitive cardiomyocytes *in vivo*.**

The expression of H3P in cardiac cells injected with adenoviruses for Cont (LacZ) or D1NLS+CDK4 (D1NLS) was examined at day 4 after manipulation. Tropomyosin (red); H3P (green); DAPI (blue). A scale bar represents 100 $\mu$m.

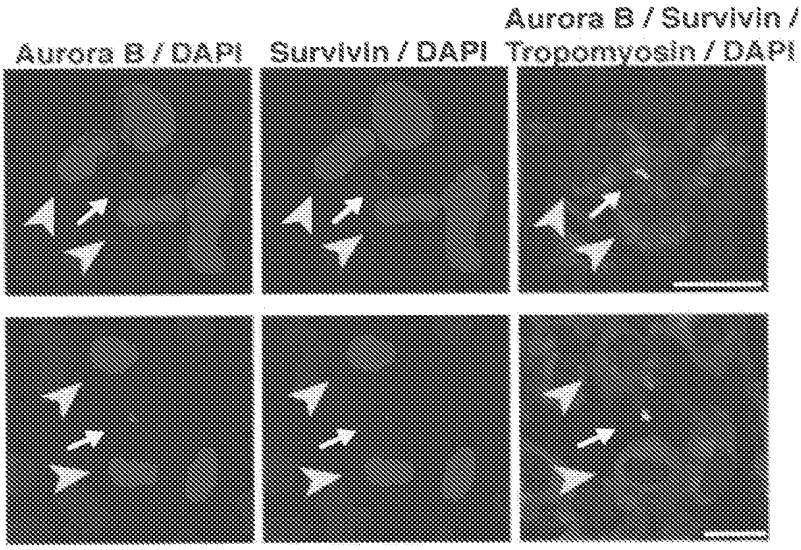


**Fig. 3 Immunostaining of Aurora B- and Survivin-postitive cardiomyocytes *in vivo*.**

The expression of Aurora B and Survivin in cardiac cells injected with adenoviruses for D1NLS+CDK4 was examined at day 4 after manipulation. Yellow arrow-heads represent two daughter cells of dividing cardiomyocytes, and a white arrow points midzone between two. Tropomyosin (red), Aurora B (green); Survivin (purple) ; DAPI (blue). A scale bar represents 10 $\mu$ m.

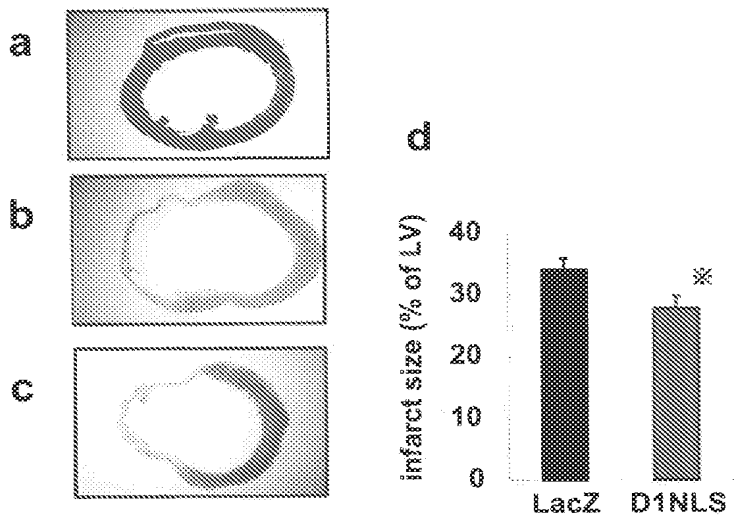


**Fig. 4 Infarct size of hearts at six weeks after ischemia/reperfusion.**

Cross-sections of hearts were stained with Masson's trichrome in sham (a), Cont/LacZ (b), and D1NLS/CDK4 (c) groups. In d, the percentage of infarct size in Cont (LacZ) or D1NLS+CDK4 (D1NLS) groups are shown. *P < 0.05, infarct size in D1NLS/CDK4 group versus Cont group.

**Table 1. Echocardiography at 6 weeks after reperfusion.**

| Group | Sham | LacZ | D1NLS |
|---|---|---|---|
| N | 10 | 11 | 12 |
| **LVDD (mm)** | 9.2 ± 0.2 | 12.7 ± 0.2* | 11.5 ± 0.2*‡ |
| **LVDS (mm)** | 6.0 ± 0.3 | 11.3 ± 0.2* | 9.6 ± 0.3*‡ |
| **Fractional Shortening (%)** | 34.8 ± 1.5 | 11.3 ± 0.9* | 16.6 ± 1.7*‡ |
| **HR (beats/min)** | 242 ± 6 | 224 ± 5 | 240 ± 8 |

Echocardiography at 6weeks after reperfusion. Each value indicates Means ± SEM. *$p<0.05$ vs. sham, ‡$p<0.05$ vs. LacZ.